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1. **Your reference** — 3.76076

2. **Patent application number** *(The Patent Office will fill in this part)* — **0202002.2**

30JAN02 E691655-27 D00027
P01/7700 0.00-0202002.2

3. **Full name, address and postcode of the or of each applicant** *(underline all surnames)*

Leiv Eiriksson Nyotek A.S.
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Norway

Patents ADP number *(if you know it)* — 8313694001

If the applicant is a corporate body, give country/state of incorporation — Norway

4. **Title of the invention** — Use

5. **Name of your agent** *(if you have one)* — Frank B. Dehn & Co.

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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London
EC4V 4EL

Patents ADP number *(if you know it)* — 166001 ✓

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Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

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Number of earlier application	Date of filing *(day / month / year)*

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11. **I/We request the grant of a patent on the basis of this application**

Signature Neil Campbell **Date** 29 January 2002

12. **Name and daytime telephone number of person to contact in the United Kingdom**

Neil Campbell
020 7206 0600

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Use

This invention relates to the use of certain polyunsaturated long-chain ketones for the treatment of psoriasis and in particular to ketones carrying electron withdrawing substituents alpha to the carbonyl functionality in such treatment.

Psoriasis is a common, chronic, inflammatory skin disorder. Psoriatic tissue is characterised by chronic inflammation in both epidermis and dermis, the disease being further characterised by hyperplasia of epidermal keratinocytes, fibroblast activation, alteration of eicosanoid metabolism, and leukocyte infiltration.

Effective treatments for psoriasis such as cyclosporin A, steroids, methotrexate and photochemotherapy all have immunosuppressive activity and are thus not ideal treatments due to their side effects. Scientists have therefore pursued other potential treatments for this disease.

It has been observed that psoriatic tissue exhibits elevated levels of arachidonic acid and eicosanoids. This suggests that phospholipase A_2 (PLA_2) may be involved in the pathogenesis of psoriasis.

The phospholipases are a group of enzymes that release unsaturated fatty acids from the sn2 position of membrane phospholipids. Once released, the fatty acids are converted by various enzymes into biologically very important signalling molecules. Release of arachidonate initiates the arachidonate cascade leading to the synthesis of eicosanoids such as prostaglandins. Eicosanoids are important in a variety of physiological processes and play a central role in inflammation. In Inflammation, Vol. 18, No.1 1994, Andersen *et al* identify the presence of certain phospholipases in psoriatic human skin.

It is therefore believed that inhibition of

phospholipase enzymes should have potential in curing some of the inflammatory symptoms, including epidermal hyperproliferation due to increased leukotriene production, related to eicosanoid production and cell activation in both epidermis and dermis in psoriasis.

In J. Chem. Soc. Perkin Trans. 1, 2000, 2271-2276 several structurally different compounds are reported as inhibitors of $cPLA_2$ in vitro. The compounds tested were based around (all-Z)-eicosa-5,8,11,14,17-pentaenoic acid (EPA) and (all-Z)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA). The paper suggests that preliminary studies show that in vitro the compounds are active as enzyme inhibitors.

The compounds in J. Chem. Soc. Perkin Trans. 1, 2000, 2271-2276 have however not been tested in vivo and more critically when devising a treatment for a disease it is necessary to ensure selectivity. There are a very large number of phospholipase enzymes known and more enzymes of this type are being discovered as medical science develops. Since phospholipases control a wide variety of different intracellular functions it is necessary to develop inhibitors of these enzymes that are selective for the particular phospholipase whose activity is to be altered. Compounds which inhibit a large number of phospholipase enzymes are of little commercial interest since the benefits of a desired enzymic inhibition will be opposed by the presence of many unwanted and potentially dangerous side effects caused by unwanted enzyme inhibitions. There remains a need therefore, to provide highly selective inhibitors of phospholipase enzymes.

The present inventors have surprisingly found that compounds of somewhat similar structure or the same structure as those identified in the Perkin Transactions paper are selective for IVa PLA enzymes and are therefore ideal candidates for the treatment of psoriasis in the absence of side effects. Given that

there are a total of 23 enzymes in the phospholipase group and each enzyme fulfils a different physiological and pathological function this is highly surprising. Moreover, seven different cystolic PLA_2 enzymes are known, one of which is the IVA PLA_2 enzyme. To be able to specifically inhibit this enzyme is very surprising.

Thus, viewed from one aspect the invention provides the use of a compound of formula (I)

R-CO-X (I)

(wherein R is a C_{16-24} unsaturated hydrocarbon group optionally interrupted α, β, γ, or δ to the carbonyl group by a heteroatom or group of heteroatoms selected from S, O, N, SO, SO_2, said hydrocarbon group comprising at least 5 non-conjugated double bonds; and

X is an electron withdrawing group)

for the manufacture of a medicament for the treatment of psoriasis.

Viewed from another aspect the invention provides a method of treating psoriasis comprising administering to an animal, preferably a mammal, e.g. human, an effective amount of a compound of formula (I) as hereinbefore described.

Viewed from another aspect the invention provides use of a compound of formula (I) as hereinbefore described for use in the manufacture of a medicament for inhibiting the enzyme IVa PLA_2.

Viewed from yet another aspect, the invention provides a pharmaceutical composition comprising a compound of formula (I) as hereinbefore described.

The group R preferably comprises 5 to 7 double bonds, preferably 5 or 6 double bonds, e.g. 5 double bonds which should be non-conjugated. It is also preferred if the double bonds do not conjugate with the carbonyl functionality.

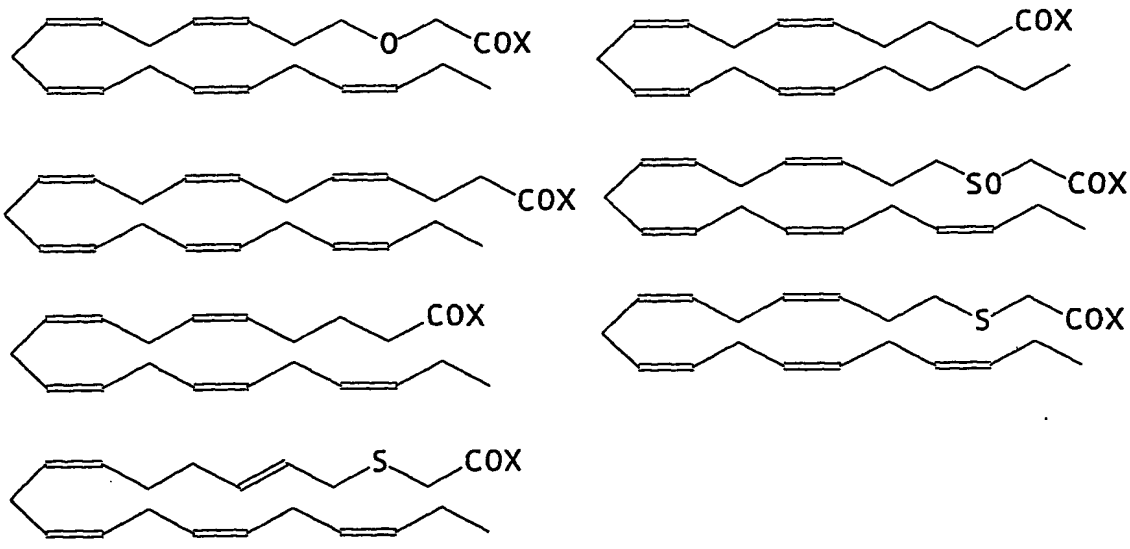
The double bonds present in the group R may be in

the cis or trans configuration however, it is preferred if the majority of the double bonds present (i.e. at least 50%) are in the cis configuration. In further advantageous embodiments all the double bonds in the group R are in the cis configuration or all double bonds are in the cis configuration except the double bond nearest the carbonyl group which may be in the trans configuration.

The group R may have between 16 and 24 carbon atoms, preferably 19 to 21 carbon atoms.

The group R may carry a heteroatom or group of heteroatoms positioned α, β, γ, or δ to the carbonyl, preferably β or γ to the carbonyl. Preferably the heteroatom is O or S or a sulphur derivative such as SO.

Specifically preferred RCOX groups are those of formula



The R group may carry up to three substituents selected from halo or C_{1-6}-alkyl. If present the substituents are preferably non-polar, and small, e.g. a methyl group. It is preferred however, if the R group remains unsubstituted.

The group X is an electron withdrawing group. Suitable groups in this regard include O-C_{1-6} alkyl, CN, CO_2-C_{1-6} alkyl, phenyl, $CHal_3$, $CHal_2H$, $CHalH_2$ wherein Hal represents a halogen, e.g. fluorine, chlorine, bromine or iodine, preferably fluorine.

In a preferred embodiment the electron withdrawing group is $CHal_3$, especially CF_3.

Highly preferred compounds for use in the invention are EPA$COCF_3$, EPA$SCOCF_3$ and AKH 217 as depicted below.

Compounds of formula (I) may be manufactured using known chemical synthetic routes. It is convenient to begin synthesis from the commercially available compounds arachidonic acid, EPA or DHA. Conversion of the acid functionality of these compounds into, for example a -$COCF_3$ group can be achieved readily, e.g. by converting the carboxylic acid into its corresponding acid chloride and reacting the same with trifluoroacetic anhydride in the presence of pyridine.

Introduction of a heteroatom into the carbon chain is also achieved readily. Conveniently, for example, the starting acid is reduced to an alcohol and, if required, converted to the corresponding thiol. The nucleophilic thiol may then be reacted with a group such as $BrCH_2COCF_3$ thereby introducing the carbonyl and electron withdrawing species. Complete synthetic protocols may be found in J. Chem. Soc., Perkin Trans 1, 2000, 2271-2276 or J. Immunol., 1998, 161, 3421.

The compounds of formula (I) may be formulated into medicaments using conventional techniques well known to the skilled pharmaceutical chemist. Thus, the compounds may be formulated with well known excipients or pharmaceutical carriers.

The medicaments of the invention may also comprise other conventional additives such as antioxidants, preservatives, colouring, flavouring etc.

The medicaments of the invention may be formulated as tablets, pills, powder, capsules, emulsions but are

preferably in the form of creams or ointments. The mode of administration may be any known mode, such as oral, nasal, transmucosal, parenteral, topical, intradermal etc. However, it is advantageous if the medicament is applied topically, i.e. directly to the infected part of the human skin.

The amount of the medicament required to effect a successful treatment will, of course, depend on the patient and on the severity of the psoriasis. The dose will be readily determined by the skilled chemist.

The compounds of the invention may be used to treat psoriasis in combination with other known pharmaceuticals for said purpose and this forms a further aspect of the invention.

The invention is described further below with reference to the following non-limiting examples and figures.

Figure 1 shows the relative inhibition of IVa PLA_2 enzyme activity for a number of compounds of the invention in comparison to commercial compounds. Recombinant IVa PLA_2 enzyme was preincubated with inhibitor (5 μM) for 10 minutes and then assayed in the mixed-micelle enzyme activity assay. The control was not pretreated with inhibitor. Results are given as % of control and are mean of duplicate determinations from 1 out of 4 representative experiments.

Figure 2 shows concentration dependent inhibition of IVa PLA_2 in the mixed-micelle enzyme activity assay. Increasing inhibition of IVa PLA_2 by $EPACOCF_3$, $EPASCOCF_3$ and $AACOCF_3$ are shown in Figure B, and increasing inhibition of IVa PLA_2 by $EPACH(OH)COCF_3$, $DHACOCF_3$ and MAFP are shown in Figure A. Results are given as % of control and are mean of duplicate determinations from one out of 2 to 4 representative experiments.

Figure 3 shows that calcium ionophore A_{23187} stimulates extracellular release of [3H]-labelled lipid in a concentration-dependent manner. HaCaT cells were

treated with A_{23187} for 1 hour, arachidonic acid and eicosanoids were extracted from cell media using Bond Elut C18 columns and contents of [3H]-labelled lipids in media were determined by scintillation counting. Each column in the figure represents the average of triplicate determinations from one out of 3 representative experiments.

Figure 4 shows that the fatty acid derivatives inhibit the extracellular release of [3H]-labelled lipid. HaCaT cells were preincubated with inhibitor (5 μM) for 1 hour and stimulated with A_{23187} (5 μM) for 1 hour. The control was treated neither with inhibitor nor A_{23187}.

Contents of [3H]-labelled lipids in media were determined by scintillation counting after octadecyl silica cartridge lipid extractions. Each column in the figure represents the average of triplicate determinations from one out of 3 representative experiments.

Figure 5 shows that TNFα or IL-1β stimulated NF-kB activation is dose-dependently inhibited by MAFP (50% and 77% respectively) or AKH217 (91% and 81% respectively).

MATERIALS AND METHODS

Materials

Calcium ionophore A_{23187}, Sigmacoat, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). Phosphatidylcholine, 1-stearoyl-2-arachidonyl and [3H] arachidonic are from Amersham (Buckinghamshire, UK). Aluminium sheets silica gel 60, ethyl acetate, iso-octane and acetic acid were purchased from Merck (Darmstadt, Germany). TNFα was a generous gift from Professor Terje Espevik, Norwegian University of Science and Technology, NTNU and IL-1β was purchased from Roche Molecular Biochemicals.

$AACOCF_3$ is from BIOMOL (Plymouth Meeting, PA, USA), and MAFP is from Cayman (Ann Arbor, MI, USA). All fatty acid compounds were stored under N_2 at -80°C.

Cell culture

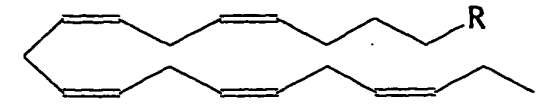
The spontaneously immortalized human skin keratmocyte cell line HaCaT was kindly provided by Prof. N. E. Fusenig (Heidelberg, Germany). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 1 g glucose/l (Gibco BRL, Life Technologies Ltd, Paisley, Scotland), supplemented with 5% fetal calf serum (FCS) (HyClone Laboratories, Inc., Utah, USA), 0.3 mg/ml L-glutamine (Sigma Chemical Company, St.Louis, Mo, USA), 0.1 mg/ml gentamicine (Sigma) and 1 µg/ml fungizone (Gibco). Confluent cells were stimulated with A_{23187}, Il-1β (10mg/ml) or TNFα (10mg/ml) in 0.5% (v/v) FCS for 1 hour before harvesting. Passages 40-80 of the cells were used. Generation of HaCat transfectants expressing luciferase under strict control of transcription factor NF-kB is described elsewhere (Anthonsen et al, J. Biol. Chem. 2001, 276, 30527). The reporter plasmid pBIIX contains two copies of the HIV NF-kB sequence cloned upstream of the mouse *fos* promoter driving expression of the *Photinus pyralis* luciferase gene.

Luciferase Assay

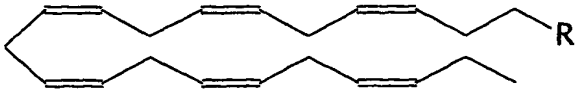
Cells were seeded in 24-round multiwell plates (2.8×10^5 cells/well). Treated cells were washed two times with phosphate-buffered saline and lysed, and luciferase activities were determined using the Luciferase Reporter Assay system (Promega) and Turner Luminometer model TD-20/20 (Turner Designs) as described by the manufacturer.

Synthesis of (all-Z)-eicosa-5,8,11,14,17-pentaenoic acid (EPA) and (all-Z)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA) derivatives

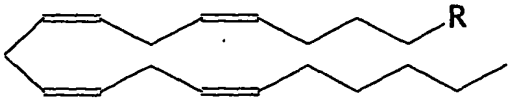
The derivatives used in the enzyme assays are shown below. $EPACOCF_3$ was prepared as described in J. Immunol., 1998, 161, 3421. AACOCF3 and MAFP were bought from suppliers as mentioned above. The remaining derivatives were prepared as described in J. Chem. Soc. Perkin Trans. 1, 2000, 2271-2276.



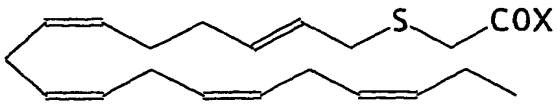
R= $COCF_3$ ⇒ $EPACOCF_3$
R= $COCH_3$ ⇒ $EPACOCH_3$



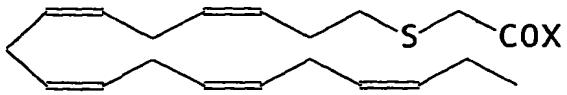
R= $COCF_3$ ⇒ $DHACOCF_3$



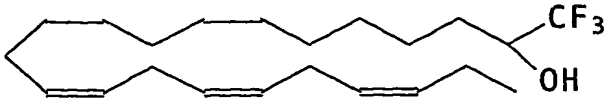
R= $COCF_3$ ⇒ AACOCF3
R= $POF\text{-}CH_3$ ⇒ MAFP



X= CF_3 ⇒ AKH217



X= CF_3 ⇒ $EPASCOCF_3$



EPACH(OH)CF_3

Mixed-micelle assay of $cPLA_2$ activity

Sources of IV PLA_2 enzyme activity were insect cells over expressing recombinant human IV PLA_2 (10 μg IV PLA_2 protein/10^6 cells; Bac PAK Baculovirus expression system; CLONTECH Laboratories, Palo Alto, CA, USA). Cytosolic fractions of insect cells were prepared as described in Schalkwijk et al (1992) Eur. J. Biochem. 210, 169-176.

The protein contents of the cytosolic fractions were measured with Bio-Rad protein assay (Bio-Rad Laboratories GmbH, Munich, Germany) using bovine serum albumin as standard. The inhibitory derivatives were added 10 minutes prior to substrate addition. The preincubation of inhibitors was performed at room temperature. IV PLA_2 enzyme activity was analyzed using [14C]-L-3 Phosphatidylcholine, 1-stearoyl-2-arachidonyl as substrate according to Wijkander et. al. (Eur. J. Biochem. 202, 873-880, 1991). After 30 min the reaction was stopped and centrifuged, the $CHCl_3$ phase was evaporated with N_2 gas to dryness and then resuspended in $CHCl_3$:MeOH (9:1, v/v). Thin layer chromatography (TLC) separated free arachidonic acid from phospholipids on aluminum sheets silica gel 60 developed with ethyl acetate: iso-octane:acetic acid: water (55: 75: 8:100, v/v/v/v) (Gronnich et al, J. Clin. Invest., 93, 1224-1233, 1994). Phosphor-Imager quantified free arachidonic acid and phospholipids, and IV PLA_2 activity was expressed as decreased arachidonic acid release by enzyme incubated with inhibitor compared to no inhibitor.

Arachidonic acid and eicosanoid detection

Confluent cells were labelled with 1 μCi/ml [^{3}H] arachidonic acid in media supplemented with 0.5 % (v/v) FCS 24 hours before cell induction and inhibition. About 90 % of the radioactive arachidonic acid were incorporated in the cell membranes. Extracellular [^{3}H] arachidonic acid was removed by washing the cells 3 times in media. The HaCaT cells were then preincubated with inhibitor for 1 hour and stimulated with calcium ionophore for 1 hour. The cell media were collected and cleared by centrifugation. Arachidonic acid and eicosanoids were extracted from media using Bond Elut C18 octadecyl columns (500 mg) (Varian SPP, Harbor City, CA) as described by Powell, Anal. Biochem. 164, 117-131,

1987; with modifications previously described Brekke, Cytokine, 4, 269-280, 1992. The samples were collected in glass tubes precoated with Sigmacoat. The ethyl acetate solution of samples were completely dried with N_2, redissolved in 0,5 ml fresh ethyl acetate and aliquots of 50 µl (triplicates) samples were subjected to liquid β-scintillation counting (Beckman LS 1701) in 5 ml Ready Protein liquid (Beckman).

The amount of PGE_2 in cell culture media from calcium ionophore stimulated HaCaT cells was measured using an enzyme immunoassay (EIA; Cayman). The assay is based on the competition between free PGE_2 and a PGE_2-acetylcholinesterase for a limited amount of PGE_2 monoclonal antibody. The media were diluted 1:10 before analyzing of the PGE_2 contents. Microplate Manager Software (Bio-Rad Laboratory) calculated the sample data.

MTT assay

Confluent cells were pretreated with inhibitors in serum free medium for 1 hour, and then treated with stimulating agent for 1 hour. Conversion of substrate [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was measured as optical density at 580 nm after 4 hours according to Mosmann [Mosmann T, J.Immunol. Methods 65, 55-63, 1983]. For each concentration of inhibitor nine parallels were measured.

RESULTS

IV PLA_2 enzyme activity

In order to investigate the action of the fatty acid derivatives as inhibitors of IV PLA_2, we measured the IV PLA_2 activity in the mixed micelle assay with recombinant IV PLA_2 as enzyme source, as described in materials and methods. The synthetic fatty acid derivatives we made are listed above, together with the commercial available

inhibitors, which we used for comparison. $EPACOCF_3$, $EPASCOCF_3$ and AKH-217 seem to have the same potency as IV PLA_2 inhibitors as $AACOCF_3$ (i.e. 75-80 % inhibition) (Figure 1). MAFP and $DHACOCF_3$ were poorer IV PLA_2 inhibitors (50% and 30% inhibition respectively). The compound $EPACH(OH)CF_3$ was also tested which results in severe attenuation of inhibitory effect (10% inhibition). $EPACOCH_3$ was made as a control compound with methyl instead of the trifluoromethyl (CF_3) group. $EPACOCH_3$ showed no inhibition (figure 1).
The IC_{50} values of $EPACOCF_3$, $EPASCOCF_3$ and $AACOCF_3$ were measured to be 2.9 ± 1.9, 3.5 ± 0 μM and 5.8 ± 1.9 μM respectively (Figure 2B). While the IC_{50} values of $DHACOCF_3$, MAFP and $EPACH(OH)CF_3$ were determined to be 21.3 ± 1.5 μM, 24± 1.4 μM and 43 ± 7.1 μM respectively (Figure 2A).

Kinetic studies with the inhibitors in the mixed micelle assay were performed in order to see if the time course was linear. A peak was achieved in two minutes (results not shown), indicating that the inhibitors are very fast acting.

In summary $EPACOCF_3$, $EPASCOCF_3$ and AKH217 seem to have similar or perhaps slightly higher potency as the commercially available compound $AACOCF_3$ in inhibiting IVa PLA_2.

Arachidonic acid and eicosanoid detection.

In order to evaluate the effect of EPA and DHA derivatives in a more biological system, we utilized the HaCaT cells as a model system [Sjursen et al, Cytokine, 12, 8, 1189-1194, 2000]. The calcium ionophore A_{23187} has been shown to induce arachidonic acid release in many cell types, probably by increasing the intracellular Ca^{2+}-concentration and thereby inducing the association of $cPLA_2$ with cellular membranes [Kramer and Sharp, FEBS Lett, 410, 49-53, 1997]. In HaCaT cell, the ionophore

induced a dose response release of [3H]-labelled arachidonic acid (Figure 3).

Concentrations higher than 10 μM of A_{23187} were toxic as determined by MTT assay.

The next step in evaluating our synthetic fatty acid inhibitors was to examine their ability to reduce the extracellular release of lipid in response to A_{23187} in HaCaT cells. Before the cell experiments were performed, we evaluated the toxicity of the inhibitors. MTT assay showed that concentrations of 25 μM and higher of the fatty acid compounds are toxic to HaCaT cells (results not shown).

[3H]AA-labelled HaCaT cells were preincubated with the inhibitors for 1 hour, and then stimulated with A_{23187} for another hour. Inhibitors used in these experiments were $EPACOCF_3$, $EPASCOCF_3$ and AKH-217. Scintillation counting of media after lipid extraction showed that fatty acid release to the media was reduced in the presence of inhibitors (Figure 4). $EPACOCF_3$ and $EPASCOCF_3$ (10 μM) reduced lipid accumulation in media by 55 %, while AKH-217 (5 μM) decreased the lipid release by 40%. The measurements of IV PLA_2 enzyme activity showed $EPACOCF_3$, $EPASCOCF_3$ and AKH217 to be as potent inhibitors as the IV PLA_2 inhibitor $AACOCF_3$ (Figure 2). Inhibition of lipid release from stimulated HaCaT cells seems to confirm $EPACOCF_3$, $EPASCOCF_3$ and AKH217 to be potent inhibitors of IVa PLA_2 (Figure 4).

In order to determine if inhibition of IVa PLA_2 has any biologic consequence, HaCaT cells were stimulated with the proinflammatory cytokines IL-1β or TNFα. As a measure of inflammation, activation of the transcription factor NF-kB was analysed. We have shown earlier that TNFα or IL-1β activates NF-kB in HaCat cells (Thommesen et al, J. Immunol., 1998, 161, 3421). NF-kB activation was analysed as luciferase expression. Treatment of the stably transfected HaCat-pBIIX cells with TNFα or IL-1β for 1 h enhanced NF-kB-dependent expression (not shown).

In the presence of inhibitors MAFP or AKH217, IL-1β stimulated luciferase expression was dose-dependently inhibited by 77% and 81% respectively. TNFα stimulated NF-kB activation was inhibited does-dependently by MAFP or AKH217 by 50% and 91% respectively (Figure 5) thus confirming that our synthetic fatty acid inhibitors may be useful in inhibiting inflammatory responses.

Claims

1. The use of a compound of formula (I)

R-CO-X (I)

(wherein R is a C_{16-24} unsaturated hydrocarbon group optionally interrupted α, β, γ, or δ to the carbonyl group by a heteroatom or group of heteroatoms selected from S, O, N, SO, SO_2 said hydrocarbon group comprising at least 5 non-conjugated double bonds; and

X is an electron withdrawing group)

in the manufacture of a medicament for the treatment of psoriasis.

2. A method of treating psoriasis comprising administering to an animal, preferably a mammal, e.g. human, an effective amount of a compound of formula (I) as hereinbefore described.

3. Use of a compound of formula (I) as hereinbefore described for use in the manufacture of a medicament for inhibiting the enzyme IVa PLA_2.

4. A pharmaceutical composition comprising a compound of formula (I) as hereinbefore described.

Figure 1

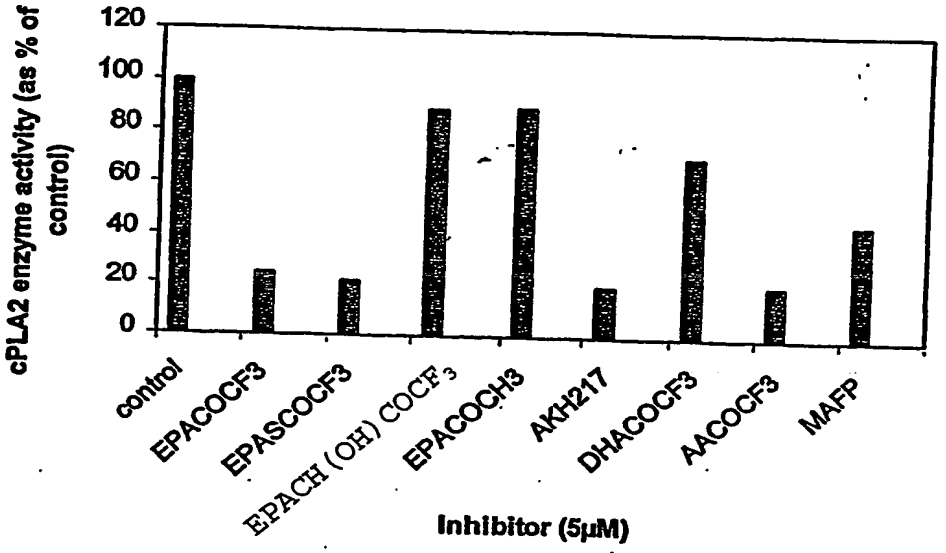
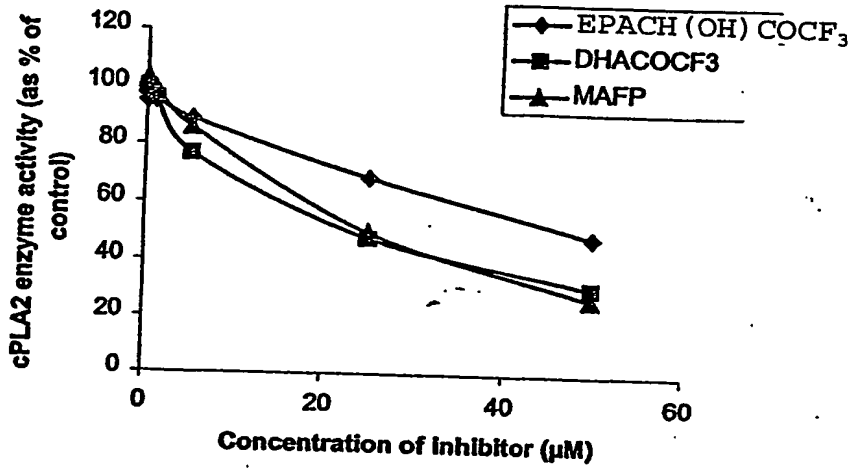

cPLA2 enzyme activity (as % of control)
120
100
80
60
40
20
0
control
EPACOCF3
EPASCOCF3
EPACH(OH)COCF3
EPACOCH3
AKH217
DHACOCF3
AACOCF3
MAFP
Inhibitor (5μM)

Figure 2 A and B



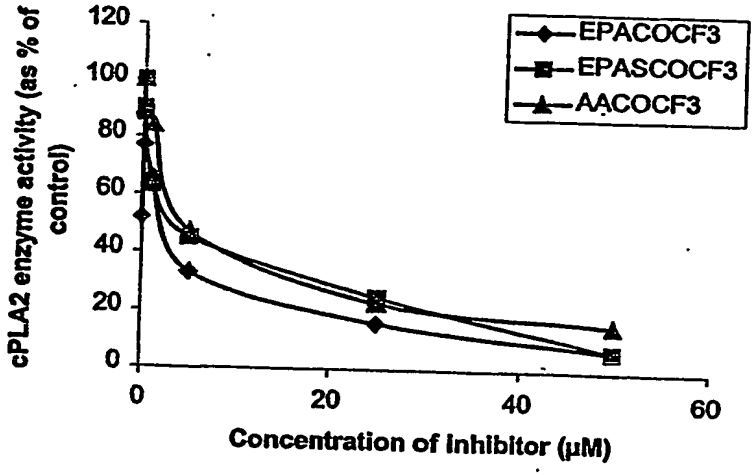


Figure 3

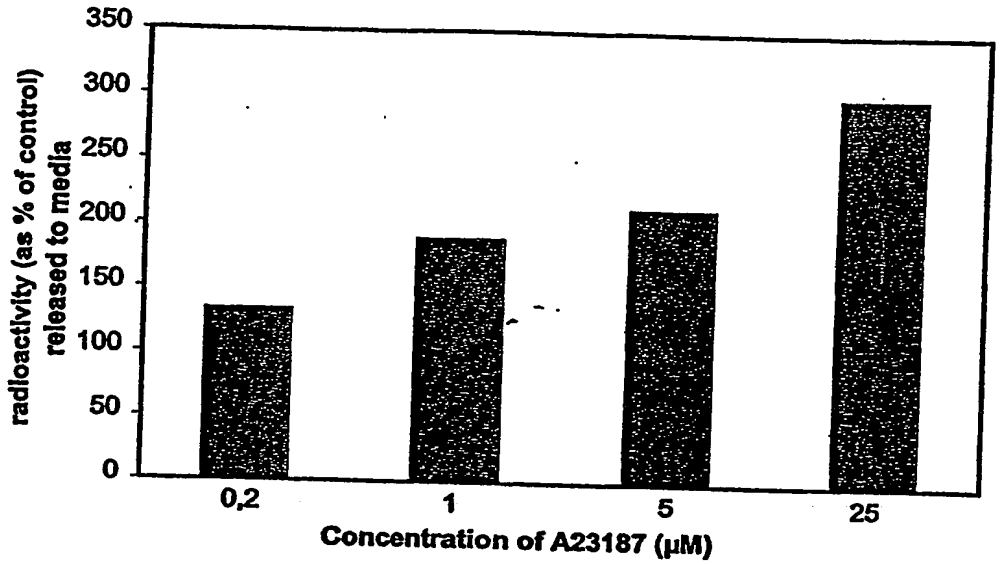
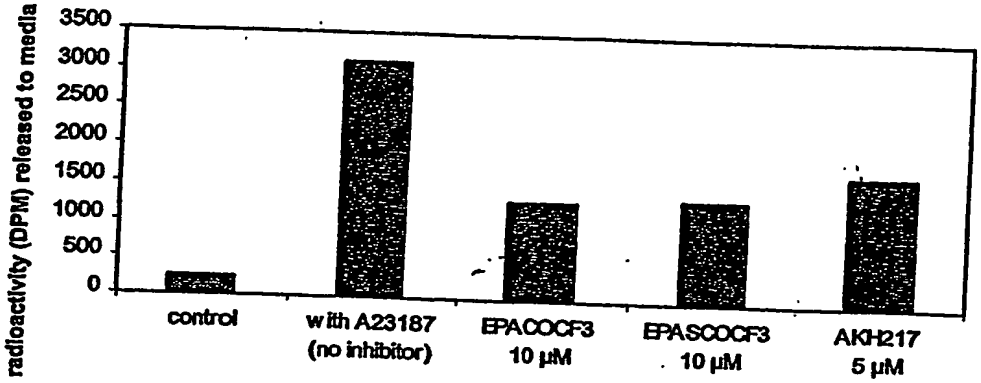

350
300
250
200
150
100
50
0
radioactivity (as % of control) released to media
0,2
1
5
25
Concentration of A23187 (µM)

Figure 4


radioactivity (DPM) released to media
3500
3000
2500
2000
1500
1000
500
0
control
with A23187
(no inhibitor)
EPACOCF3
10 µM
EPASCOCF3
10 µM
AKH217
5 µM

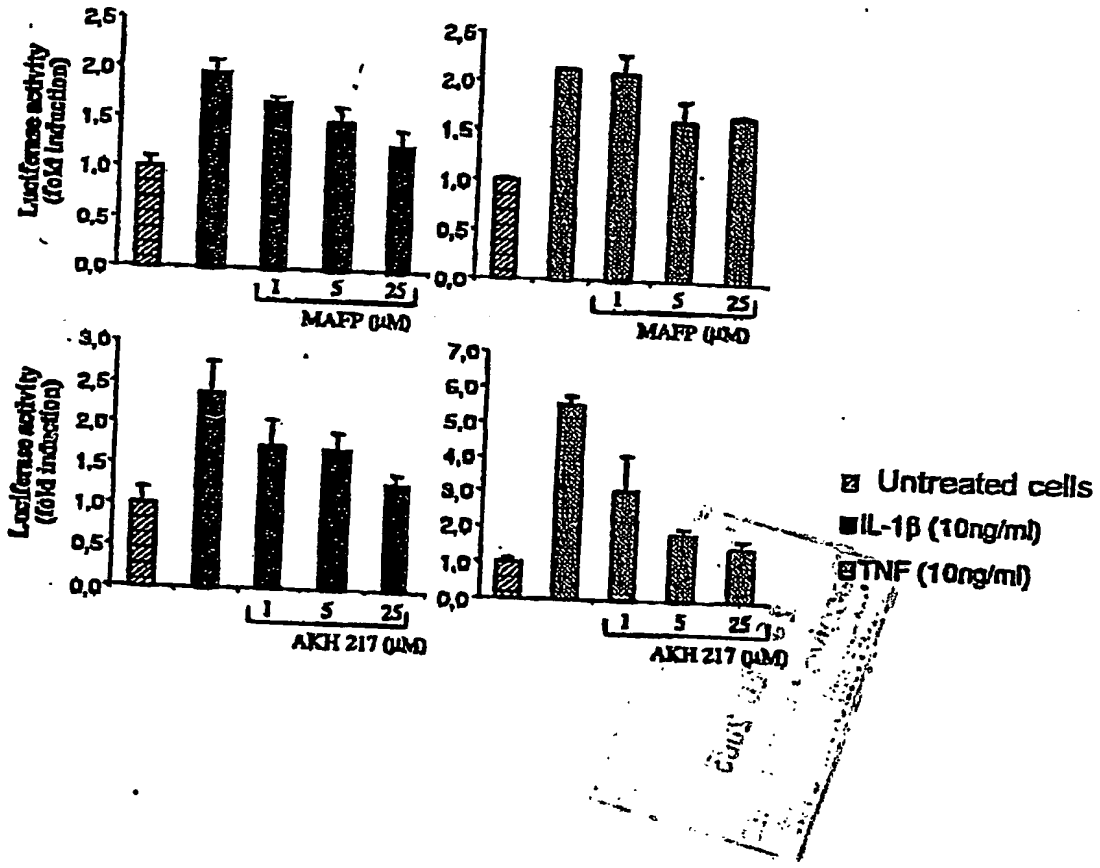

Luciferase activity (fold induction)
2,5
2,0
1,5
1,0
0,5
0,0
1
5
25
MAFP (µM)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
AKH 217 (µM)
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Untreated cells
IL-1β (10ng/ml)
TNF (10ng/ml)

Figure 5